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Prolactin inhibits a major tumor-suppressive function of wild type BRCA1

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ABSTRACT

Even though mutations in the tumor suppressor, BRCA1, markedly increase the risk of breast and ovarian cancer, most breast and ovarian cancers express wild type BRCA1. An important question is therefore how the tumor-suppressive function of normal BRCA1 is overcome during development of most cancers. Because prolactin promotes these and other cancers, we investigated the hypothesis that prolactin interferes with the ability of BRCA1 to inhibit the cell cycle. Examining six different cancer cell lines with wild type BRCA1, and making use of both prolactin and the growth-inhibiting selective prolactin receptor modulator, S179D PRL, we demonstrate that prolactin activation of Stat5 results in the formation of a complex between phospho-Stat5 and BRCA1. Formation of this complex does not interfere with nuclear translocation or binding of BRCA1 to the p21 promoter, but does interfere with the ability of BRCA1 to transactivate the p21 promoter. Overexpression of a dominant-negative Stat5 in prolactin-stimulated cells resulted in increased p21 expression. We conclude that prolactin inhibits a major tumor-suppressive function of BRCA1 by interfering with BRCA1's upregulation of expression of the cell cycle inhibitor, p21.

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Introduction

Mutations in Breast Cancer 1, BRCA1, have been implicated in the development of multiple cancers, including those of the breast, ovary and prostate [1–4]. People with BRCA1 mutations have a much higher incidence of tumor development [5], and BRCA1 knockout in mouse models causes tumor formation [6]. BRCA1 has several different tumor-suppressive functions, including DNA damage repair, transcriptional regulation, and ubiquitination [7]. For this study, our focus was on the ability of BRCA1 to transactivate the cyclin dependent kinase inhibitor, CDKN1A (p21). Increased expression of p21 results in G1/S phase arrest and may eventually lead to apoptosis [8]. Given the clinical importance of BRCA1 as a tumor suppressor (5), it is surprising that most tumors have wild type BRCA1: in a recent study examining 41 human breast cancer cell lines, over 90% exhibited wild type BRCA1 [9], and similar results were reported in ovarian tumors [10,11]. Because the level of expression of BRCA1 can be illustrative of chemoresistance through DNA damage repair [12], it seems that the entire function of wild type BRCA1 is not lost during the formation of many tumors. How there can be elevated BRCA1 with an intact DNA repair function, but a lost or overwhelmed anti-proliferative function, remains unclear.

Prolactin is a peptide hormone best known for its ability to promote milk production [13]. It is also an important growth factor for most tissues, and many cancer cells produce prolactin as an autocrine growth factor [14]. When binding to its receptor, prolactin signals through multiple pathways, including the Jak2/Stat5 pathway, resulting in both proliferation and differentiation. Activated Stat5 has been reported to be associated with tumor progression in some, but not all studies (reviewed in [15]). In breast cancers, constitutively-activated and nuclear-localized Stat5 was found in 76% of invasive breast adenocarcinomas [16]. In prostate cancers, hyper-activated Stat5 was associated with higher histological grade [17]. Thus, regulation of prolactin–Stat5 signaling may be crucial in cancer transformation.

BRCA1 is expressed in high amounts in proliferating cells, presumably to ensure repair of any damaged DNA [18]. However, under other circumstances, induction of BRCA1 leads to upregulation of p21 and inhibition of the cell cycle [8]. In the mammary gland, as demonstrated by work from the Chodosh laboratory, the expression of BRCA1 is increased under circumstances when prolactin is proliferative, and decreased when prolactin is more differentiative [19]. A change in the role of prolactin from a hormone that promotes proliferation to one that promotes differentiation can be achieved through interaction with different receptor isoforms or by post-translational modification of prolactin: Thus, we and others have produced direct and associative evidence that higher expression

Abbreviations: PRL, prolactin; LFPRLR, long form prolactin receptor; SF1bPRLR, short from 1b prolactin receptor; S179DPRL, a selective prolactin receptor modulator.

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levels of long form prolactin receptor (LFPRLR) promote proliferation and disease progression, while more short form 1b prolactin receptor (SF1b PRLR) expression reduces proliferation and favors differentiation in mammary [20–23] and other [24,25] tumor cells. In addition, unmodified prolactin stimulates cell growth while a mimic of phosphorylated prolactin, the selective PRLR modulator S179DPRL, inhibits cell growth and promotes differentiation both in vitro and in vivo [25–29]. Thus, we initially hypothesized that the two forms of the PRLR and prolactin and S179D PRL would have opposite effects on the expression and function of BRCA1, an hypothesis that was proven incorrect. Instead, we demonstrate that prolactin activation of Stat5 results in the formation of a complex between phospho-Stat5 and BRCA1 that inhibits the function of BRCA1 at the p21 promoter.

Materials and methods

Cell culture and treatment

All cell lines were originally purchased from ATCC and were authenticated utilizing Short Tandem Repeat (STR) analysis. Cells were routinely cultured in RPMI 1640 medium supplemented with 10% FBS and were used for experiments with passage number <20. Six human cancer cell lines were chosen and their cancer type and BRCA1 and p53 status are listed in Table 1. 5×10^5 cells were treated with prolactin or S179DPRL at 100 ng/mL for 24, 48 and 72 hours and the expression of BRCA1 was determined. Most experiments used non-transfected cells, but transfection was required for the promoter analysis, and to assess the result of increased expression of LFPRLR or SF1b PRLR.

Plasmid construction

The full length (2400 bp) p21 promoter construct was a gift from L.P. Freedman [30]. The different lengths of the p21 promoter region were amplified by PCR, ligated to TA vector, digested with restriction enzymes and cloned into pGL4.17 vector (Promega, cat #E6721). Primer information is included in Table 2. The 1390 bp p21 promoter construct was digested from the 1439 bp p21 promoter construct using DraI digestion and then self-ligated to form the 1390 bp p21 promoter construct. This construct was missing the fragment from –84 to –126 (cut by DraI) where the BRCA1-interacting element was present. The 1224 bp plasmid was constructed by generating a XhoI cutting site at 1227 bp through PCR mutagenesis and then digesting with XhoI to remove the fragment between 2400 bp and 1224 bp.

The Stat5a 740 bp truncation construct was characterized by Yamashita et al. and Wang et al. [31,32] and was shown to act as a dominant negative of Stat5a. The amino acids after 740 were deleted from Stat5a.

Table 1
Cell lines and status of BRCA1 and p53. Wt, wildtype; NHS, no hotspot mutations.

Cell line	Tissue type	ATCC number	Some characteristics
T-47D	Breast cancer	HTB-133	p53 mutant, BRCA1 wt
MCF-7	Breast cancer	HTB-22	Both p53 and BRCA1 wt
PC3	Prostate cancer	CRL-1435	p53 null, BRCA1 wt
TOV-112D	Ovarian cancer	CRL-11731	p53 mutant, BRCA1 NHS
OV-90	Ovarian cancer	CRL-11732	p53 mutant, BRCA1 NHS
TOV-21G	Ovarian cancer	CRL-11730	p53 wt, BRCA1 NHS

Table 2
Primers used for cloning.

2400 bp	A gift from Freedman L.P. [30].
1439 bp	F: AGGAGAAAGAAGCCTGTCCT R: GCAGCTGCTCACACCTCAGC
1224 bp	F: GTTTCAGGCACAGACTCGAGGCAAAGGTGAAGTCCAGG R:CCTGGACTTCACCTTTGCCTCGAGTCTGTGCCTGAAAC
840 bp	F: TCCTGGCCAACAAAGCTGCT R: GCAGCTGCTCACACCTCAGC
143 bp	F: CGCTGGGCTAGCCAGG R: CCCAAGCTTAGCTGCTCACACCTCAGC
Stat5a 740 bp	Forward: GGGGATCCATGGCGGGCTGGATCCAG Reverse: CCGCTCGAGTCACTGTGGGTACATGT

Immunoprecipitation

Cells were incubated in serum free RPMI1640 for 24 hours to synchronize the cells and lower background signals and then treated with 100 ng/mL PRL or S179DPRL for 24 hours and harvested using 100 μL RIPA lysis buffer. The protein concentration was quantified using Bio-Rad protein assay (Bio-Rad, cat# 500-0006). Protein lysates (200 μg) were first precleared with 5 μL normal rabbit IgG and then immunoprecipitated with 5 μL rabbit polyclonal BRCA1 antibody (Santa Cruz sc-646) and precipitated with 100 μL protein A/G beads (Pierce #20421). The beads were then washed 5 times with IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2) and eluted with 50 μL Elution buffer (0.1 M Glycine–HCl, pH 2.5). The eluate was adjusted to physiological pH by adding 10 μL Neutralization buffer (1 M Tris, pH 9) per 100 μL eluate. The eluate was analyzed by Western blot.

Nuclear and cytoplasmic extraction

One milliliter of cytoplasmic extraction buffer (1 M Hepes, 3 M KCl, 0.25 M EDTA, 10% NP40) was added, and the cells were scraped and transferred to a 1.5 mL microcentrifuge tube. After centrifugation for 1 min at 14,000 rpm at 4 °C, the supernatant (cytoplasmic fraction) was collected. The pellet was resuspended in 100 μL ice cold cytoplasmic extraction buffer, mixed by pipetting up and down and incubated on ice for 10 min before centrifugation for 10 min at 13,000 *g* at 4 °C. Nuclear extraction buffer (1 M Hepes, 3 M NaCl, 0.25 M EDTA) of 80 μL was then added to the pellet and the pellet was resuspended. This suspension was shaken for 10 min at 4 °C, and then centrifuged for 10 min at 13,000 *g* at 4 °C. The pellet was separated from the supernatant (Nuclear fraction). Both cytosolic and nuclear fractions were subjected to Western blot or immunoprecipitation analysis.

Chromatin immunoprecipitation

Cells were seeded in 10 cm dishes and cultured at 37 °C until 80% confluent. Medium was changed to serum-free and cells were incubated for 24 hours before prolactin treatment. Then, 100 ng/mL of prolactin or S179DPRL was added for 20 hours. Cells were then washed with 10 mL Dulbecco's PBS (twice) and fixed by adding 10 mL DPBS containing 270 μL 37% formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding 1 mL 1.25 M glycine for 5 min at 25 °C. Cells were washed and the pellet was resuspended in 1 mL CHIP sonication buffer plus protease inhibitors (1% Triton X-100, 0.1% Deoxycholate, 50 mM Tris 8.1, 150 mM NaCl, 5 mM EDTA). Samples were sonicated, centrifuged at 13,000 *g* for 15 min at 4 °C to remove debris, and the supernatant was retained and precleared using 1–5 μg IgG plus 40 μL protein A/G beads for 30–60 min. ChIP buffer (10% Triton X-100, 1.9% EDTA, disodium dehydrate, 1% SDS) was added to a final volume of 1 mL for each sample. To this was added 10 μl BSA (10 μg/μl) with thorough mixing. Each sample was divided into Input, anti-BRCA1 and anti-rabbit IgG. ChIP buffer without Triton (800 μL) and 5 μg antibody (BRCA1/rabbit IgG) were added to tubes and nutated at 4 °C for 2 hours. Protein A/G beads were washed before use and then 40 μL was added to each tube along with 2 μL herring sperm DNA (10 mg/mL) to avoid nonspecific DNA binding and nutated at 4 °C for 2 h. Samples were centrifuged at 400 *g* for 2.5 min at 4 °C and the supernatants stored at –20 °C. Beads were washed with 1 mL cold ChIP buffer without protease inhibitors by inverting the sample to suspend the resin then pelleting again, as before, with removal of supernatants. Following two further washes, the pelleted and drained beads were resuspended in 250 μL of Elution buffer (1 mL 10% SDS, 2 mL 0.5 M $NaHCO_3$, 10 μL sperm DNA) and placed on a nutator at 25 °C for 15–20 min. To each were added 40 μl 2.5 M NaCl followed by placement in a 65 °C bath for 4 h. The precipitated DNA fragment was purified using gel extraction (Sigma Genelute Gel extraction NA1111-1KT). The eluate was then used for PCR.

Western blot

Depending on the size of target protein, SDS-PAGE gels were either high percentage (12%) to analyze small proteins such as p21 or low percentage (7%) to detect large proteins such as BRCA1. Gels were run at 100 V (constant) and transferred onto nitrocellulose paper using a standard semi-dry method. Blots were then blocked with 5% BSA at 25 °C for one hour and incubated with 1st antibodies overnight at 4 °C. Blots were then washed and incubated with HRP-linked 2nd antibody against species of 1st antibody for 1 hour at 25 °C and then developed. Antibodies used in this study are listed in Table 3.

Cell cycle analysis

TOV-112D and T47D cells were synchronized in serum-deprived medium for 24 hours followed by treatment with DPBS or 100 ng/mL prolactin or S179DPRL for another 24 hours. Cells were collected and stained with propidium iodide (eBioscience 00–6990) following the manufacturer's instructions. Cell cycle was analyzed by flow cytometry using FlowJo software.

Table 3
Antibodies used.

Name	Supplier and cat. #	Dilution used
p21	Santa Cruz sc-397	1:1,000 in 5% BSA with 0.02% sodium azide
BRCA1	Santa Cruz sc-646	1:200 in 5% BSA with 0.02% sodium azide
Stat5a	Santa Cruz sc-1081	1:1,000 in 5% BSA with 0.02% sodium azide
pStat5a	Santa Cruz sc-101806	1:1,000 in 5% BSA with 0.02% sodium azide
p-ERK	Santa Cruz sc-7383	1:1,000 in 5% BSA with 0.02% sodium azide
ERK 1/2	Santa Cruz sc-94	1:1,000 in 5% BSA with 0.02% sodium azide
pAkt	Cell Signaling #4060X	1:1,000 in 5% BSA with 0.02% sodium azide
Akt	Santa Cruz sc-5298	1:1,000 in 5% BSA with 0.02% sodium azide
Actin	Santa Cruz sc-1016	1:10,000 in 5% BSA with 0.02% sodium azide
Goat anti-rabbit HRP	Sigma A0545	1:40,000 in 10 mL TBST buffer
Goat anti-mouse HRP	Santa Cruz sc-2062	1:10,000 in 10 mL TBST buffer

Statistical analyses

All experiments were conducted a minimum of 3 times using a minimum of triplicates on each occasion. All cell lines were used for all analyses, with the exception of the receptor transfection experiments that were confined to the low endogenous receptor expressing cell line, PC3. Statistical significance was determined by ANOVA with post tests and Bonferroni corrections for multiple comparisons, where applicable. A p value < 0.05 was considered significant.

Results

BRCA1 levels were increased by both prolactin and S179DPRL

Since prolactin promotes proliferation and S179D PRL inhibits proliferation and promotes differentiation [25–29,33], and there was some indication in the literature that BRCA1 increases during proliferation and decreases during differentiation [19], we hypothesized that prolactin and S179D PRL would have opposite effects on BRCA1 levels. Contrary to expectations, BRCA1 levels were increased in response to both prolactin and S179DPRL. This occurred in all six cancer cell lines tested, although the degree and the peak time of induction varied in different cell types. Fig. 1 shows examples of breast

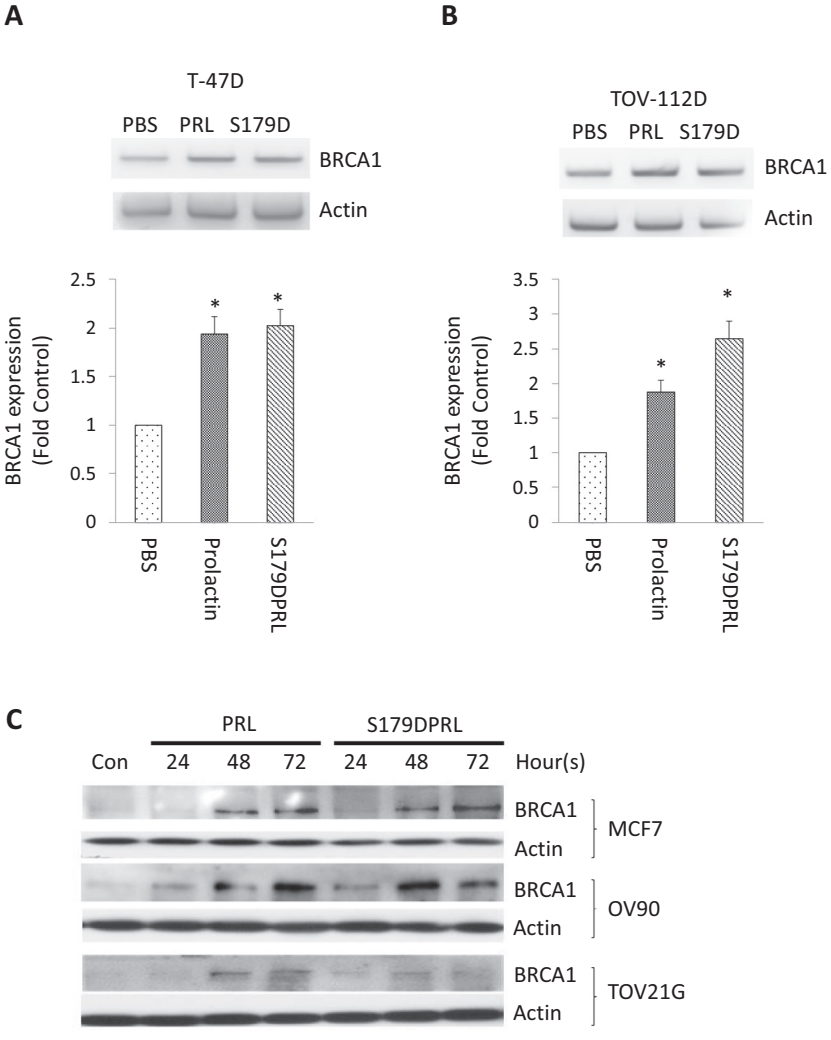

Fig. 1. Both prolactin and S179DPRL elevate BRCA1. (A, B) Western blots and quantification of same demonstrating an elevation in BRCA1 in response to both prolactin and S179DPRL in T47D (A) and TOV-112D (B) cells. Cells were seeded in 6 well plates and treated with 100 ng/mL prolactin or S179DPRL for 48 hours. Values were normalized to β-actin. *$p < 0.05$. (C) Western blots showing the time course of induction of BRCA1. Cells were seeded in 6 well plates and treated with 100 ng/mL prolactin or S179DPRL for 24, 48, 72 hours. Vehicle control cells shown were from the 0 hour time point. Controls were assayed at 0, 24, 48, 72 hour time point and there was no difference in BRCA1 expression.

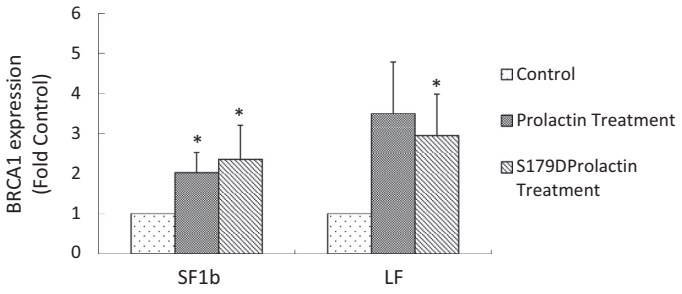

Fig. 2. The isoform of the PRLR is irrelevant to the response. Quantification of Western blot after PC3 cells were transfected with either LFPRLR or SF1b PRLR and treated with 100 ng/mL prolactin or S179DPRL for 72 hours. Values normalize to β-actin (*$p < 0.05$).

(MCF7, T-47D) and ovarian (OV90, TOV-112D, and TOV-21G) cancer cell lines and the quantification of the response in T-47D and TOV112D cells and Fig. 2 shows the response in prostate cancer (PC3) cells.

Since different spliced PRLR isoforms mediate signaling to proliferation or inhibition of proliferation and promotion of differentiation [20–25], we then asked whether the different time courses and degrees of BRCA1 induction in the different cell lines were the result of differential PRLR isoform expression. The PC3 cell line was chosen for PRLR isoform transfection experiments because the low levels of endogenous expression of PRLRs allowed for transfection of the receptors without excessive overexpression. Even though endogenous receptor expression is low, the receptors in PC3 cells have nevertheless been shown to respond to prolactin by activating downstream signaling pathways [34]. Fig. 2 not only quantifies the PC3 response, but also shows that increased expression of the proliferative (LFPRLR) or the anti-proliferative (SF1b PRLR) isoform of the receptor led to an increase in BRCA1 levels in response to either prolactin or S179DPRL. Thus, even though the short receptor (SF1b PRLR) lacks a large portion of the intracellular signaling domain, it could mediate the ability of both ligands to increase BRCA1 levels. Furthermore, because there was no difference in the result between the two forms of receptor, differential ratios of LFPRLR to SF1b PRLR expression would not explain the differential time course or degree of response among the cell lines.

BRCA1 induction by prolactin and S179DPRL in terms of p21 elevation

To evaluate the impact of prolactin and S179DPRL on the downstream function of BRCA1, expression of the cell cycle inhibitor, p21, was examined. Consistent with the anti-proliferative role of S179DPRL, BRCA1 induced by S179DPRL was able to increase p21 protein. Also, in agreement with the proliferative function of prolactin, BRCA1 induced by prolactin was not capable of increasing p21. Fig. 3 shows examples from a breast, ovarian and prostate cancer cell line, and panel C shows that the result was unchanged in PC3 prostate cancer cells overexpressing either LFPRLR or SF1b PRLR. To be sure that receptor transfection did not distort the results, induction of p21 protein was also examined in non-transfected cells. As shown in Fig. 4, induction of p21 in response to S179DPRL was dose-related in TOV-112D and PC3 cells, but seemed not to reach a maximum in T47D cells, perhaps as a result of expression of a larger number of PRLRs. By contrast, there was no effect of prolactin at any dose in the breast, ovarian and prostate cancer cell lines.

Induction of p21 is also reflected in the effect on the cell cycle. With S179DPRL-treated TOV-112D, there were no cells in S phase and the percentage of cells in G1 was higher than control or prolactin-treated. Also, 12.5% of cells were undergoing apoptosis after

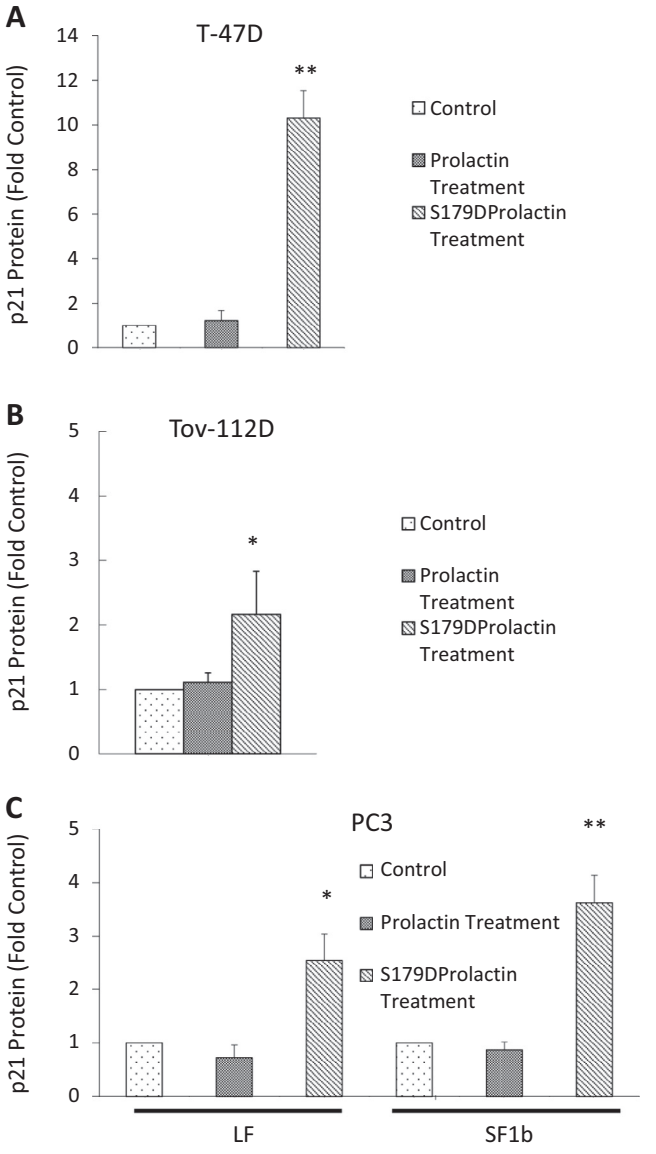

Fig. 3. S179DPRL, but not prolactin, increased p21 expression even though both elevated BRCA1. Cells were seeded in 6 well plates and treated with 100 ng/mL prolactin or S179DPRL for 72 hours. p21 expression was then analyzed by Western blot and normalized to β-actin in T47D cells (A), Tov-112D cells (B) and PC3 cells overexpressing either LFPRLR or SF1bPRLR (C) (**$p < 0.01$; *$p < 0.05$).

exposure to S179DPRL. In the more slowly-growing T47D cells, the effects after 24 hours of treatment were more subtle, but included an increase in G1, as appropriate to the action of p21 (Fig. 5).

Expression of p21 is regulated by a variety of factors and the promoter region has many potential binding sites for transcription factors, including BRCA1 (Fig. 6A). To determine which transcription factors were involved in the induction of p21, we made a series of constructs containing different regions of the p21 promoter upstream of luciferase, ranging from the full length promoter to 143 bp (Fig. 6A). To eliminate the confounding influence of p53, PC3 cells, which have null p53, were chosen to conduct the promoter analysis. PC3 cells transfected with different constructs and the SF1b PRLR were then analyzed after treatment with prolactin or S179DPRL for 20 hours.

With the full length p21 promoter or other constructs containing the BRCA1 response element (93–133 bp on the p21 promoter) [8], an S179DPRL stimulus increased luciferase expression. In addition, as one would expect in this cell line, removal of both p53

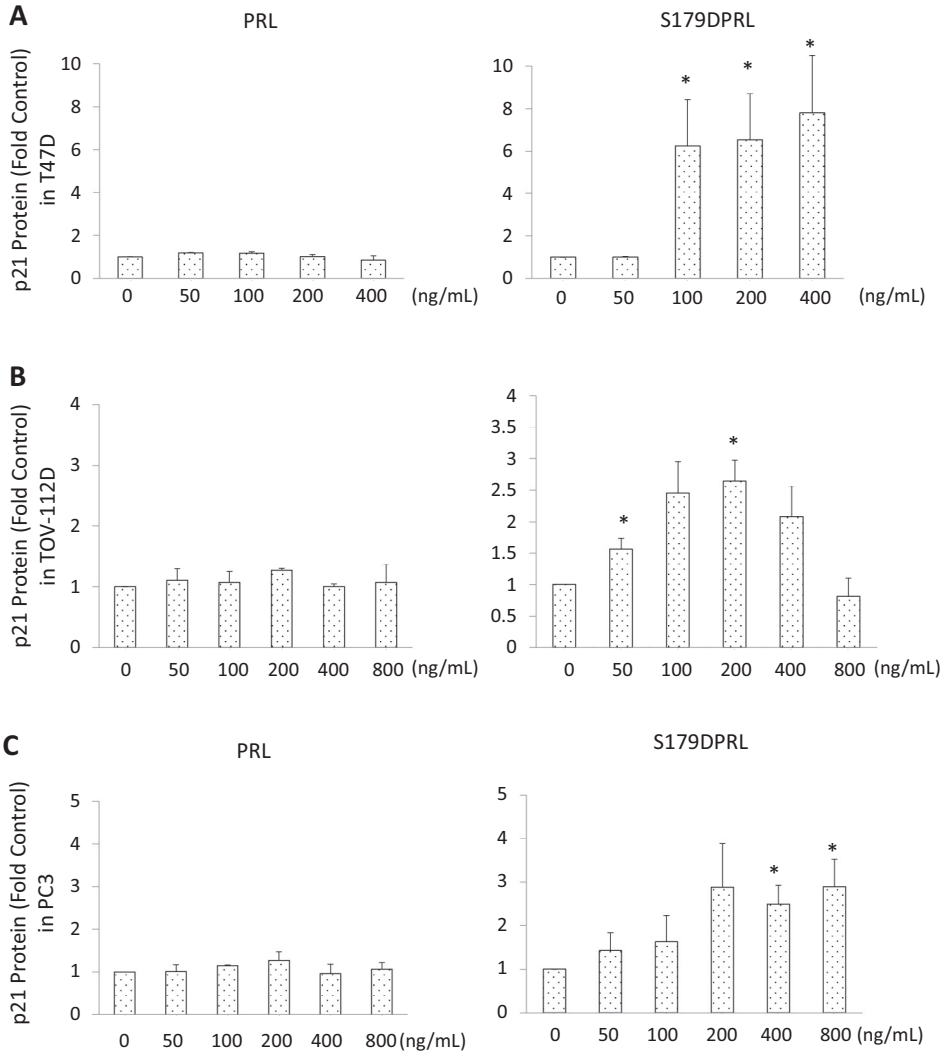

Fig. 4. The response to S179DPRL is dose-related. (A) T47D, (B) TOV-112D and (C) non-transfected PC3 cells were treated for 48 hours, and subjected to quantitative Western blot analysis normalized to β-actin (*$p < 0.05$).

elements had no effect (1439 bp, 1224 bp and 840 bp constructs). Most interestingly, the minimal promoter (143 bp) containing only the BRCA1 response element and a basic TATA box was still able to drive luciferase expression in response to S179DPRL. On the other hand, when the BRCA1 response element was removed while keeping all others intact (1390 bp), the expression of luciferase went down to unstimulated levels, thereby unequivocally indicating BRCA1 involvement in p21 induction in response to S179DPRL (Fig. 6B). In comparison with the results with S179DPRL, the prolactin-induced BRCA1 did not increase luciferase activity in cells transfected with the same constructs (data not shown); the luciferase values were the same as with no addition of prolactin. This implied functional interference with BRCA1 when cells were stimulated with prolactin.

The activated signaling molecule, p-Stat5, formed a complex with BRCA1 and interfered with its p21 transactivation function

In questioning how S179DPRL increased levels of BRCA1 and p21, and yet prolactin, while increasing levels of BRCA1, had no effect on p21 expression, we considered the differences in signaling between the two ligands. Among the 5 signaling molecules examined (Erk, Stat5, Akt, AMPK and PKC), activation of Stat5 was completely different with prolactin versus S179DPRL in all cell lines. Consistent with previous results, prolactin activated Stat5 whereas S179DPRL did not (Fig. 7, showing examples of T47D and PC3 cells with endogenous high and low expression of PRLRs, respectively).

A mechanism through which activation of Stat5 might interfere with BRCA1 would be for activated Stat5 and BRCA1 to form a complex. To test whether there was an interaction between activated Stat5 and BRCA1, co-immunoprecipitation was performed using a BRCA1 antibody and Western blotting with anti-p-Stat5. Fig. 8A shows the result with the human breast cancer cell line, T-47D. This cell line (as all other cell lines used in this study) expresses autocrine prolactin. As seen in Fig. 8A, both prolactin treatment (cells exposed to both exogenous and autocrine) and control (cells exposed to autocrine) showed complex formation between p-Stat5 and BRCA1. This did not occur with S179DPRL treatment. Thus, S179DPRL not only did not activate Stat5, but also inhibited the ability of autocrine prolactin to activate Stat5.

There are three possible ways formation of a complex between p-Stat5 and BRCA1 might interfere with the ability of BRCA1 to increase p21 expression: (1) complex formation might prevent entry of BRCA1 into the nucleus, (2) complex formation might prevent binding of BRCA1 to the p21 promoter, or (3) once in the nucleus and bound to the promoter, complex formation might prevent transactivation of the promoter. To determine which of these was

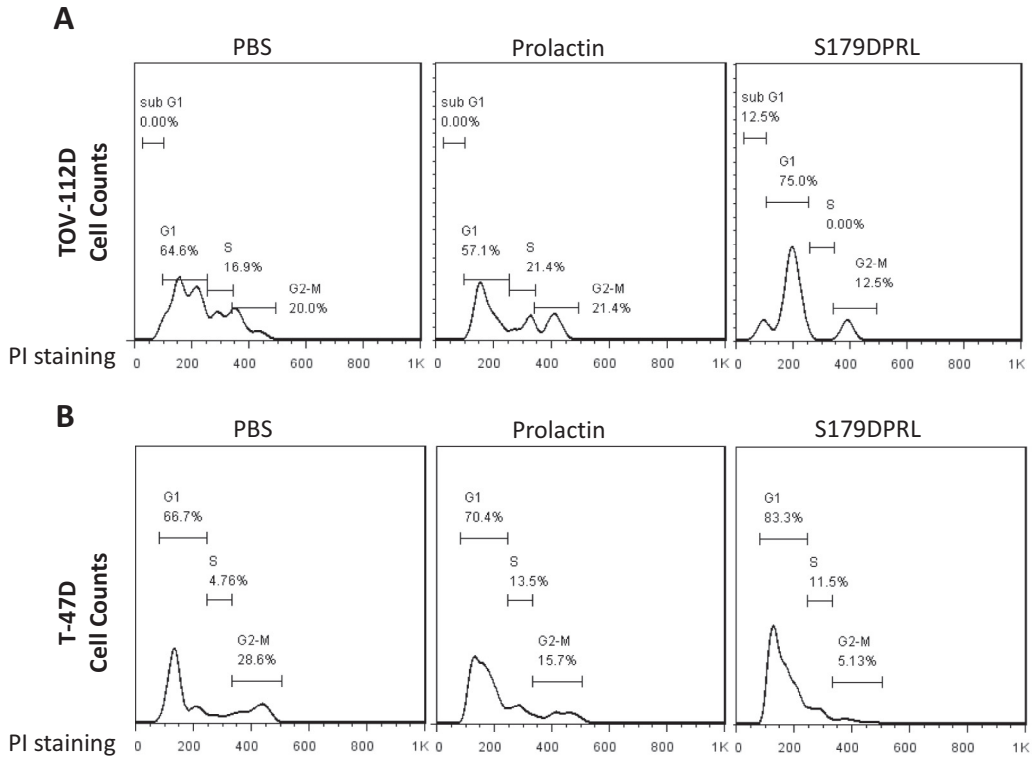

Fig. 5. Cell cycle analysis after treatment with prolactin or S179DPRL. Cells were synchronized by a 24 hour incubation in serum-free medium and then given prolactin or S179DPRL (100 ng/mL) for 24 hours prior to staining with propidium iodide. (A) TOV-112D cells and (B) T-47D cells.

the case, cells were treated with prolactin and the cytosolic and nuclear fractions were isolated and subjected to immunoprecipitation with anti-BRCA1, followed by immunoblotting with anti-p-Stat5. As shown in Fig. 8B, the BRCA1–p-Stat5 complex was located in the nucleus, indicating no effect on entry into the nucleus. To examine the interaction of BRCA1 with the p21 promoter after prolactin and S179DPRL treatment, whole cell chromatin was crosslinked and immunoprecipitated with anti-BRCA1 antibody and the interaction of BRCA1 with the p21 promoter was examined (Fig. 8C). There was no difference between prolactin or S179DPRL treatment of cells in the binding of BRCA1 to the p21 promoter. This left us with the possibility that p-Stat5–BRCA1 complex formation interfered with the transcription of p21. To determine whether this was the case, we constructed the dominant negative form of Stat5a described by Yamashita et al. and Wang et al. [31,32]. With overexpression of this dominant negative, non-phosphorylatable form of Stat5a, treatment with prolactin was now able to drive p21 expression. Thus, prolactin's activation of Stat5 interferes with BRCA1's p21 transactivation function (Fig. 8D). To determine whether this interference was specific for prolactin or common to several activators of Stat5, two additional activators of Stat5, human IL-2 and human growth hormone, were tested for their ability to both induce BRCA1 expression and activate the minimal p21 promoter. While treatment with growth hormone or IL-2 increased expression of BRCA1 (Fig. 8E), there was no induction of luciferase driven by the minimal p21 promoter (Fig. 8F and G). Furthermore, growth hormone antagonized the effect of S179DPRL (Fig. 8F).

Discussion

Contrary to our initial hypothesis, we found the form of the PRLR associated with proliferation as well as the form associated with inhibition of proliferation to mediate increased levels of BRCA1. Thus, we can conclude that it is not differential ratios of these two forms of the receptor that regulate the level of BRCA1 or responsiveness to ligand. Rather, the responsiveness to ligand in terms of increased BRCA1 seems related to overall expression levels of all PRLRs. For example, non-transfected PC3 cells with low levels of PRLR responded the least well and T47D cells with high levels of PRLR responded most robustly. The LFPRLR and SF1b PRLR have very different intracellular signaling domains [22]. Because both forms of the receptor transduced the signal leading to increased levels of BRCA1, we know that the signaling pathway is common to both receptor isoforms, and from the work of Nelson et al. this is most likely via Akt [35].

Again contrary to our initial hypothesis, both prolactin and S179DPRL induced BRCA1 expression. Thus, whether we examined receptors or ligands with opposite effects on proliferation, both versions of each increased BRCA1 levels. It therefore does not appear that the overall level of BRCA1 in a cell dictates whether or not cell proliferation occurs.

Even though both ligands elevated levels of BRCA1, only with S179D PRL did increased levels of BRCA1 lead to an increase in the downstream cell cycle-regulating molecule, p21. Since p21 is downstream of either p53 or BRCA1 [36], use of a cell line with non-functional p53 for the promoter analysis demonstrated that p53 was not required for S179DPRL-mediated p21 induction. This was further demonstrated by the lack of effect of removal of p53 response elements from the promoter. By contrast, absolute dependence on the BRCA1 response element was demonstrated by equivalent responses by the full length and 143 bp minimal promoter and the lack of activity of the full length promoter minus the BRCA1 response element. These results emphasize the importance of direct interaction of BRCA1 with the p21 promoter.

A previous study in our laboratory indicated that S179DPRL led to an increase in vitamin D receptor (VDR) expression in human

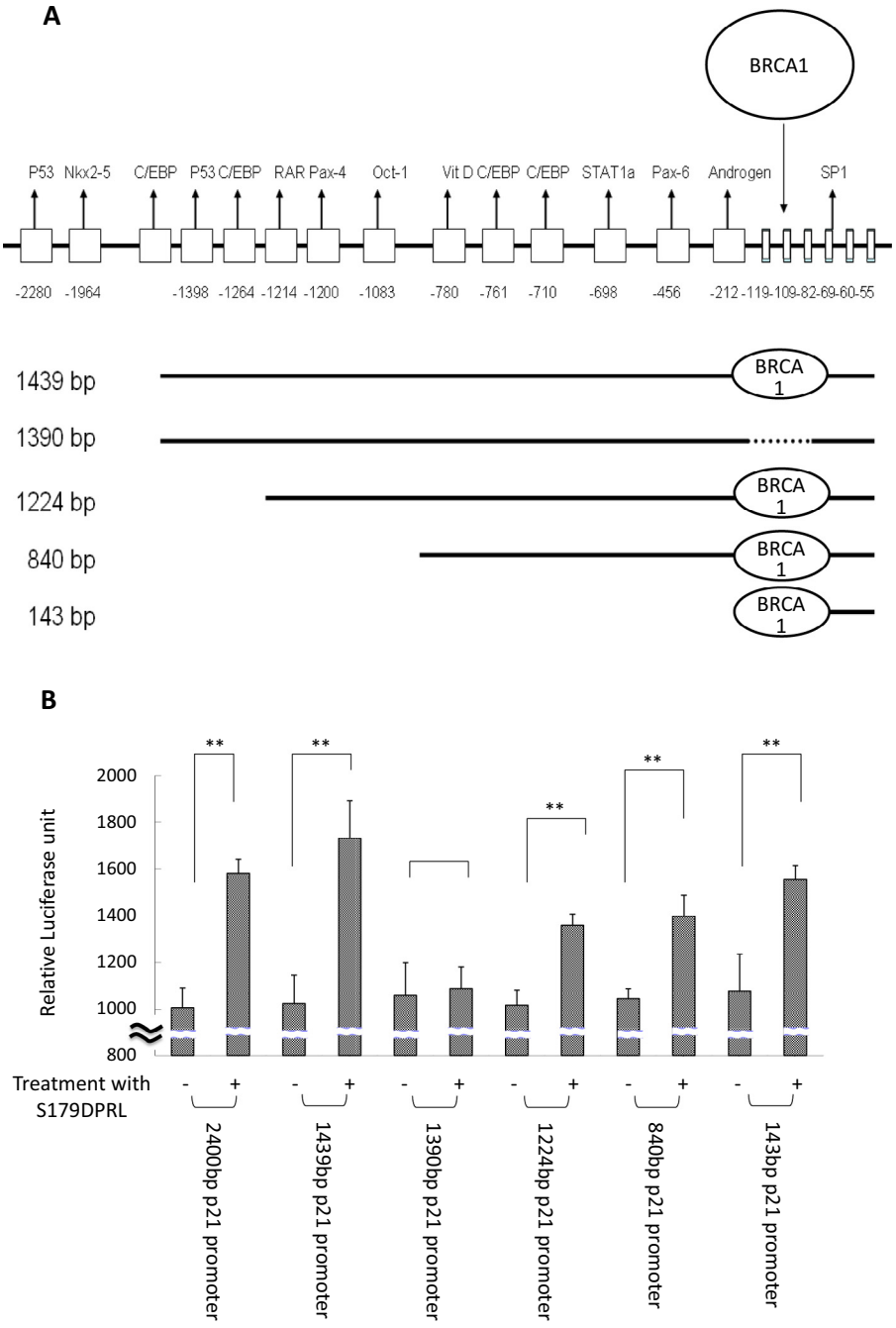

Fig. 6. p21 promoter luciferase constructs and their relative response to S179DPRL. (A) Different constructs with deletions of the p21 promoter indicating possible transcription factor interacting sites. (B) PC3 cells were co-transfected with SF1b PRLR, a plasmid containing the different constructs of p21 promoter, and a plasmid expressing β-galactosidase for normalization. After 4 hours transfection, cells were treated with 100 ng/mL prolactin, S179DPRL or vehicle for 20 hours. The data presented are normalized to the control transfected with all the same constructs but without any treatment (**$p < 0.01$; *$p < 0.05$).

prostate and mouse mammary cells. The VDR in turn interacted with the p21 promoter leading to an elevation of p21 [26,37,38]. However, as we now show, removal of the BRCA1 response element, while keeping the VDR response element, eliminated the response to S179DPRL. Thus, while the VDR is important, BRCA1 is essential.

In osteosarcoma cells, we have previously shown that prolactin blocks nuclear translocation of the VDR through the promotion of formation of a complex between the VDR and BRCA1 [39]. Given that prolactin also increases the level of BRCA1, whether or not formation of a complex with the VDR would contribute to the effect of prolactin on BRCA1 function in any cell line would depend on the relative expression levels of BRCA1 and the VDR.

By contrast to the VDR–BRCA1 complex, formation of a complex between p-Stat5 and BRCA1 does not affect nuclear translocation of BRCA1. Instead, the transactivation function of BRCA1 was hindered. Since there is no Stat5 binding motif within the 2400 bp p21 promoter, and removal of the BRCA1 response element eliminates the response, binding of the BRCA1–p-Stat5 complex to the p21 promoter likely occurs through BRCA1 (Fig. 9).

While both forms of the PRLR transduce a signal leading to increased BRCA1 levels, only the LFPRLR activates Stat5 [33]. Thus

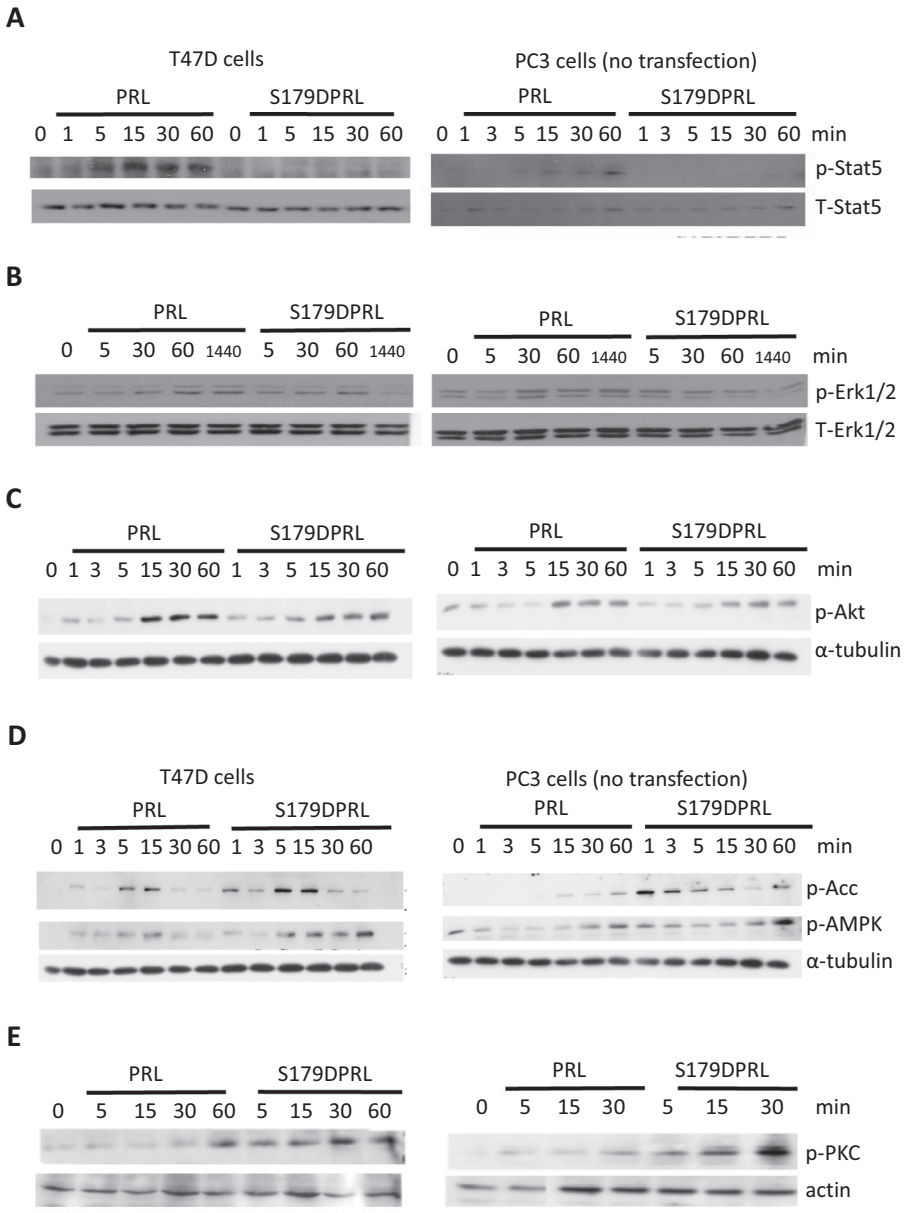

Fig. 7. All signaling molecules except Stat5 were activated by both prolactin and S179DPRL. Responses to 100 ng/mL prolactin or S179DPRL at various time points are shown in T47D and PC3 cells by Western blots. There was no detectable activation of Stat5 with S179DPRL treatment (A). However, (B) MAPK/ERK (C) Akt (D) AMPK and (E) PKC were activated by both prolactin and S179DPRL.

increased expression of the LFPRLR increases the ability of prolactin to activate Stat5, which would in turn decrease the ability of BRCA1 to transactivate p21, thereby allowing increased cell proliferation in response to prolactin. The crucial nature of activated Stat5 in this process was demonstrated by the use of the dominant negative Stat5.

In addition to endocrine and autocrine prolactin, Stat5 signaling is initiated by other ligands that may contribute to prolonged activation during tumor development. IL-2, for example, is one of five cytokines overexpressed in breast cancer, but not detected in normal mammary tissues [40]. Both IL-2 and the IL-2R have been detected in malignant breast tumors, and cell proliferation is increased by exogenous IL-2 [41]. In prostate cancers, expression of the IL-2R is also upregulated compared to normal tissue [42]. Autocrine and endocrine growth hormones have also been implicated in the development and progression of a variety of cancers [43] and expression of the growth hormone receptor is upregulated in cancers [44,45]. Stat 5 activation by any one or all of these hormones/cytokines may contribute to inhibition of BRCA1's p21-related activities.

At the same time, each of the Stat5-activating ligands tested increased total BRCA1 levels. Given the correlation between BRCA1 levels and chemoresistance in tumors with wild type BRCA1 [12] and the ability of both prolactin and growth hormone to increase chemoresistance [46], it does not seem likely that the formation of a complex between BRCA1 and p-Stat5 has any inhibitory effect on DNA repair, although this remains to be directly examined. If Stat5 activation is important to chemoresistance and S179DPRL inhibits Stat5 activation, then S179DPRL could potentially inhibit DNA repair. Treatment of cancer cells with other prolactin antagonists has certainly made them more susceptible to chemotherapeutics [47] and S179DPRL alone induces apoptosis ([25], and data herein). However,

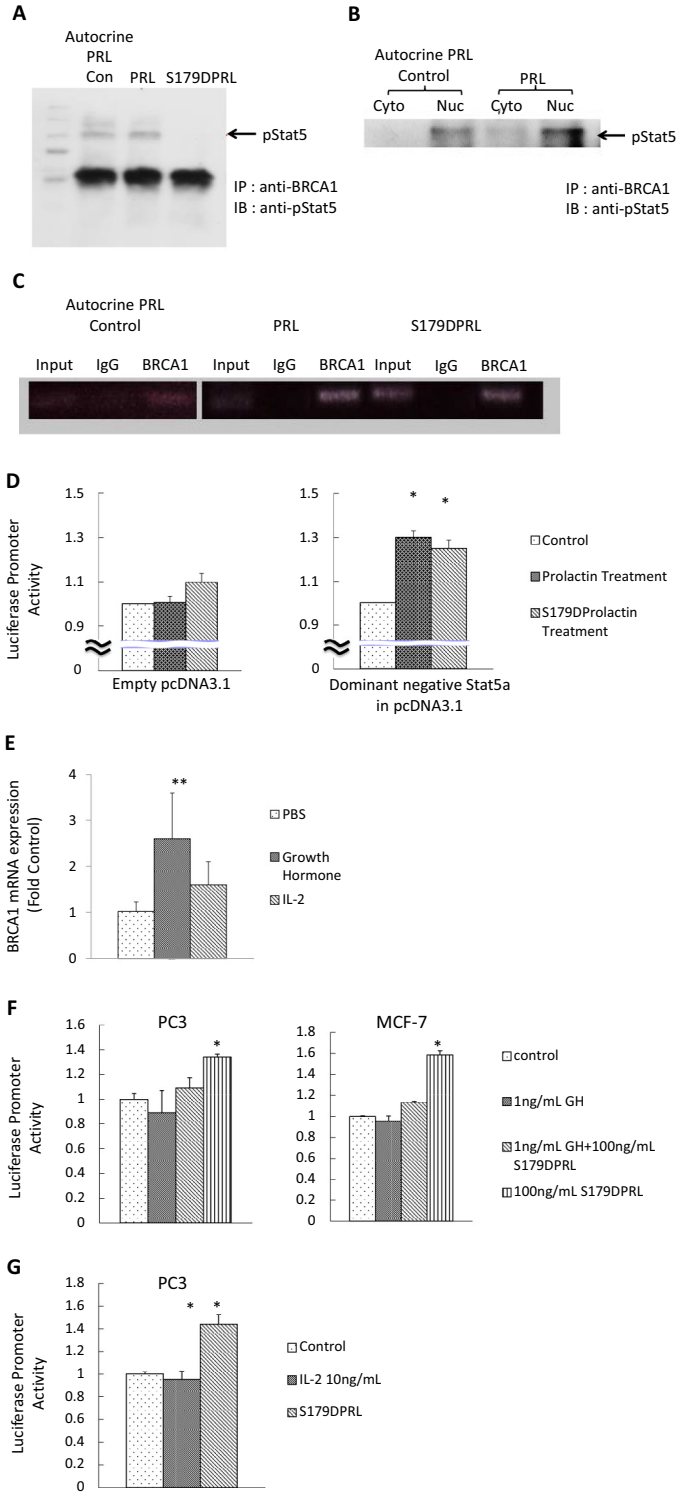

Fig. 8. Complex formation between BRCA1 and p-Stat5 negates transactivation of p21. (A) Immunoprecipitation with antibody against BRCA1in T-47D cells treated with 100 ng/mL prolactin or S179DPRL for 30 min and Western blotting with antibody against p-Stat5. (B) Nuclear localization after subcellular fractionation. (C) Chromatin immunoprecipitation with anti-BRCA1 or non-specific IgG after 20 hours of treatment with the same doses. (D) p21 promoter activity after transfection with dominant negative Stat5a. (E) BRCA1 induction by growth hormone and IL-2. (F) p21 promoter activity in response to growth hormone, S179DPRL or a combination for 20 hours. (G) p21 promoter activity in response to IL-2, or S179DPRL (**$p < 0.01$; *$p < 0.05$).

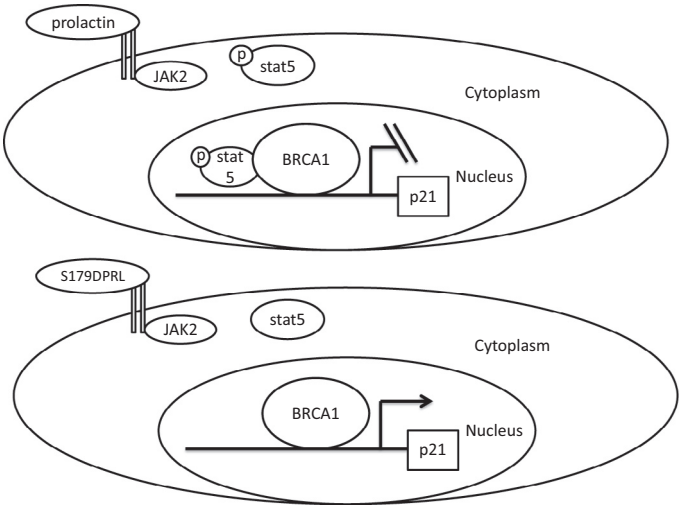

Fig. 9. Working model. Prolactin treatment (upper panel) causes Stat5 activation, which in turn forms a complex with BRCA1 and translocates into nucleus where it binds to the p21 promoter, but fails to drive transcription. By contrast, S179DPRL (lower panel) does not activate Stat5 and therefore does not form a complex between Stat5 and BRCA1, allowing activation of the promoter.

without experimentation, the balance of a variety of reported effects is difficult to predict. This is because S179DPRL elevates p21 and p21 in turn has been shown to play several different roles in DNA repair. Thus, cells expressing mutant p21 are deficient in nucleotide excision repair [48]. Furthermore, p21 is recruited and co-localized to sites of DNA damage with other repair factors upon irradiation, suggesting a role in double strand repair [49]. On the other hand, p21 inhibits the activity of PARP-1 in base excision repair [50].

Prolactin is produced in several modified forms [51], one of which, the phosphorylated form, is able to block the activation of Stat5 signaling [22,52]. In this study, we used the selective PRLR modulator, S179DPRL, which is a mimic of the phosphorylated hormone [22,29,33]. It blocks Stat5 signaling, and treatment with either S179DPRL or a dominant negative form of Stat5 led to an elevation of p21 in the presence of autocrine or exogenous prolactin. This is consistent with previously described opposite roles for unmodified and phosphorylated prolactin in the development of cancer [20,25–27,33].

Most importantly, the results presented here explain deficient anti-proliferative BRCA1 functioning in tumor cells expressing wild type BRCA1. While the pathway we have uncovered would be important to allow for physiological proliferation in response to prolactin, prolongation of the prolactin stimulus may lead to excess proliferation and/or the reduced ability to undergo apoptosis, and hence to tumor development.

We conclude that prolactin and other hormones/cytokines that activate Stat5 can have a major impact on tumor development by interfering with the cell cycle regulatory function of wild type BRCA1.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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